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# *Ralstonia solanacearum* Dps Contributes to Oxidative Stress Tolerance and to Colonization of and Virulence on Tomato Plants[▽]

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***Ralstonia solanacearum*, an economically important soilborne plant pathogen, infects host roots to cause bacterial wilt disease. However, little is known about this pathogen's behavior in the rhizosphere and early in pathogenesis. In response to root exudates from tomato, *R. solanacearum* strain UW551 upregulated a gene resembling Dps, a nonspecific *D*NA binding *p*rotein from *s*tarved cells that is critical for stress survival in other bacteria. An *R. solanacearum dps* mutant had increased hydrogen peroxide sensitivity and mutation rate under starvation. Furthermore, *dps* expression was positively regulated by the oxidative stress response regulator OxyR. These functional results are consistent with a Dps annotation. The *dps* mutant caused slightly delayed bacterial wilt disease in tomato after a naturalistic soil soak inoculation. However, the *dps* mutant had a more pronounced reduction in virulence when bacteria were inoculated directly into host stems, suggesting that Dps helps *R. solanacearum* adapt to conditions inside plants. Passage through a tomato plant conferred transient increased hydrogen peroxide tolerance on both wild-type and *dps* mutant strains, demonstrating that *R. solanacearum* acquires Dps-independent oxidative stress tolerance during adaptation to the host environment. The *dps* mutant strain was also reduced in adhesion to tomato roots and tomato stem colonization. These results indicate that Dps is important when cells are starved or in stationary phase and that Dps contributes quantitatively to host plant colonization and bacterial wilt virulence. They further suggest that *R. solanacearum* must overcome oxidative stress during the bacterial wilt disease cycle.**

Bacterial wilt caused by *Ralstonia solanacearum* is a lethal disease affecting diverse economically important crops worldwide (20). The pathogen attacks over 200 species in more than 50 plant families (21). Although known primarily as a soilborne plant pathogen, *R. solanacearum* also survives in soil, water, and latently infected plants (20). The bacterium typically invades its host through natural or mechanical root wounds, multiplies in the root cortex, and then rapidly colonizes the xylem, where it reaches high cell densities. Once wilt symptoms develop, plants usually die, releasing the pathogen back into the soil (42).

*R. solanacearum* is a tropical bacterium adapted to warmer climates, with the exception of a clonal group belonging to phylotype II, sequevar 1, of the *R. solanacearum* species complex (13). This group, historically and for regulatory purposes known as race 3 biovar 2 (R3bv2), causes brown rot of potato and bacterial wilt of tomato in tropical highlands and some temperate zones (11, 41, 45, 46). Because of its virulence at relatively cool temperatures, R3bv2 is a quarantine pest in Europe and Canada and a select agent pathogen in the United States (27).

*R. solanacearum* virulence is quantitative and complex, with many contributing factors such as type II-secreted proteins, type III-secreted effectors, extracellular polysaccharide, and several plant cell wall-degrading enzymes (16, 17, 36, 38). Much of what is known about *R. solanacearum* comes from studies focusing on mid- or end-stage disease caused by tropical or warm-temperate strains (8, 30). A few virulence factors are known to function early in disease development: motility, energy taxis, and type IV pili, which collectively direct the bacterium toward and facilitate attachment to the host root (26, 44, 49, 50). However, *R. solanacearum* traits that contribute to fitness and virulence in the rhizosphere are not well understood for either tropical or R3bv2 strains.

In soil, bacteria experience environmental stressors, such as pH and temperature extremes and water and oxygen limitation, as well as competition for nutrients (47). Plant roots release exudates and sloughed-off cells, supplying sufficient energy to sustain large microbial communities, provided other nutrients such as N, P, and Fe are present (19, 47). While rhizosphere bacteria can enjoy rapid growth in this relatively rich environment, fluctuating nutrient availability means that soil-dwelling microbes must survive periods of starvation (47).

*R. solanacearum* also encounters oxidative stress in the rhizosphere. Plant roots produce reactive oxygen species (ROS) in response to many stimuli (25, 32). Several studies implicate ROS in root development and in interactions between roots and microbes (5, 24). We previously found that during plant colonization *R. solanacearum* is exposed to host-derived ROS, which triggers a bacterial oxidative stress response that adapts the pathogen to the xylem environment and is necessary for full virulence (8, 14).

We previously described an *in vivo* expression technology (IVET)-like screen that identified *R. solanacearum* genes upregulated in the tomato rhizosphere (12). These genes encoded several known bacterial wilt virulence factors, such as the type 3 secretion regulator HrpG, the type IV pilus assem-

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Description[a] | Source or reference |
|---|---|---|
| Strains | | |
| *Escherichia coli* DH5α | F$^-$ *endA1 relA* ϕ80d*lacZ*ΔM15 *hsdR17 supE44 thi-1 recA1 gyrA96* | Invitrogen |
| *Ralstonia solanacearum* | | |
| UW551 | Wild-type race 3, biovar 2 | 48 |
| UW551*cheW rex-5* | UW551*cheW*::*aacC1 rex-5*(*dps*::*gus*); Gm$^r$ Km$^r$ | 12 |
| UW551*dps* | UW551*dps*::*aacC1*; Gm$^r$ | This study |
| UW551*dps*(pUFJ10) | UW551*dps* carrying vector pUFJ10 | This study |
| UW551*dps*(pUF*dps*) | UW551*dps*::*aacC1* complemented with pUFJ10*dps*; Gm$^r$ Km$^r$ | This study |
| UW551rif | UW551 spontaneous Rif$^r$ mutant | 41 |
| UW551*oxyR* | UW551*oxyR*::*aacC1*; Gm$^r$ | This study |
| Plasmids | | |
| pSTBlue-1 | Cloning vector; Ap$^r$ Km$^r$ | Novagen |
| pUCGm | pUC with *aacC1*; Gm$^r$ | 39 |
| pST*dps* | 1.6-kb UW551 fragment including RRSL_03240; Ap$^r$ Km$^r$ | This study |
| pST*dps*::Gm | 0.85-kb Gm$^r$ cassette in PshA1 site of RRSL_03240 | This study |
| pUFJ10 | Cosmid vector; Gm$^r$ Km$^r$ | 15 |
| pUFJ10*dps* | PCR-amplified 1.6-kb fragment including UW551 RRSL_03240; Gm$^r$ Km$^r$ | This study |

[a] Gm$^r$, gentamicin resistance; Km$^r$, kanamycin resistance; Ap$^r$, ampicillin resistance; Rif$^r$, rifampin resistance.

bly protein PilP, global virulence regulator VsrA, and early virulence regulator PehR. The screen further identified a high-affinity cytochrome *c* oxidase necessary for *R. solanacearum* growth in microaerobic conditions (12). This paper presents our analysis of another rhizosphere-induced gene that encodes Dps, a *D*NA binding *p*rotein from *s*tarved cells originally described in *Escherichia coli* (2). Dps belongs to a family of ferritin-like stress-induced proteins that bind nonspecifically to DNA in stationary-phase bacteria (2, 29, 40). In *E. coli*, Dps helps maintain DNA integrity under environmentally challenging conditions, including starvation, oxidative damage, pH shock, and thermal stress (2, 10, 18, 29, 33). Dps also protects the soilborne plant-associated bacteria *Agrobacterium tumefaciens* and *Pseudomonas putida* from oxidative stress (9, 37).

Traits that adapt *R. solanacearum* to detrimental conditions in the rhizosphere are likely to be important for pathogenic success. We hypothesized that Dps is required for adaptation to nutrient and oxidative stress and, thus, for bacterial wilt disease development. We found that Dps was highly expressed after starvation and contributed to oxidative stress tolerance in starved *R. solanacearum* cells. Furthermore, this protein was necessary for wild-type bacterial wilt disease development and for colonization of tomato xylem, suggesting that the bacterium must overcome a nutrient-poor and/or oxidative environment in the rhizosphere and xylem of host plants.

## MATERIALS AND METHODS

**Bacterial strains, media, and growth conditions.** Strains and plasmids used in this study are listed in Table 1. All *R. solanacearum* strains used in this study were derived from the wild-type R3bv2 strain UW551 (48). *R. solanacearum* strains were grown at 28°C in Casamino Acids-peptone-glucose (CPG) broth or plates (22). Boucher's minimal media (BMM) (7) with glucose was used when minimal medium was required. *E. coli* strains were grown at 37°C using Luria-Bertani (LB) (31) solid or liquid medium supplemented with appropriate antibiotics. Antibiotics were added to media at final concentrations of 25 mg liter$^{-1}$ kanamycin, 5 mg liter$^{-1}$ gentamicin, 4 mg liter$^{-1}$ nalidixic acid, and 25 mg liter$^{-1}$ rifampin. Unless otherwise noted, medium components were obtained from Difco Laboratories (Detroit, MI) and chemicals were from Sigma-Aldrich (St. Louis, MO).

**DNA manipulation and sequence analysis.** Standard methods were used to isolate, digest, amplify, and clone DNA (4). *E. coli* and *R. solanacearum* were transformed by electroporation as previously described (1). DNA and protein sequences were analyzed using SoftBerry (SoftBerry, Inc., Mount Kisco, NY), Biology Workbench (http://workbench.sdsc.edu/), NEBcutter (New England Biolabs, Ipswich, MA), and the Integrated Microbial Genomes database (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). DNA sequencing was performed at the University of Wisconsin—Madison Biotechnology Center (Madison, WI).

**Quantitative β-glucuronidase (Gus) activity assays.** To measure expression levels of *dps*::*gus* gene fusions, strains were grown under nutrient stress conditions as described below, and 1.1-ml samples were taken at intervals. Bacterial population size was determined by dilution plating (0.1 ml). Gus activity in the remaining sample was measured as described previously (8), with activity calculated as nmol 4-methylumbelliferone released per minute per cell.

**Mutagenesis of *dps* in UW551.** RRSL_03240, the *dps* locus, was mutated using marker-assisted gene replacement. Briefly, an ~1.6-kb region, including RRSL_03240 and flanking DNA, was amplified from UW551 genomic DNA using primers 5′-AGATCACCGTGGAACCTGTC-3′ and 5′-AAGCGCTTGA AGAACGGATA-3′. The product was A-T cloned into pSTBlue-1 to create pST*dps*. The *aacC1* cassette from pUCGm (39), which codes for gentamicin resistance, was blunt-end cloned into the pST*dps* PshAI site using Klenow exonuclease, creating pST*dps*::Gm. This plasmid, which cannot replicate in *R. solanacearum*, was electroporated into UW551, and double-recombined transformants were selected by gentamicin resistance and kanamycin sensitivity. The expected mutation in UW551 was confirmed by PCR analysis and the resulting strain was named UW551*dps*. A complementation plasmid, named pUF*dps* (Km$^r$), was created by blunt-end cloning the *dps* insert into EcoRI-digested pUFJ10, which is stably maintained in *R. solanacearum* (15). pUF*dps* was electroporated into competent UW551*dps* cells to create the complemented strain UW551*dps*(pUF*dps*).

**Oxidative stress response assay.** Sensitivity of *R. solanacearum* strains to ROS was determined by spreading a lawn of bacterial culture on a plate and measuring the zone of growth inhibition around a filter disk saturated with hydrogen peroxide ($H_2O_2$) (14). Briefly, overnight broth cultures of wild-type UW551, mutant UW551*dps*, and complemented UW551*dps*(pUF*dps*) were pelleted, washed, and resuspended in BMM buffered with 20 mM morpholineethanesulfonic acid (MES) to pH 6.5. To measure sensitivity of stationary-phase cells, the cell density of overnight cultures was not adjusted. To measure sensitivity of exponential-phase cells, overnight cultures were diluted and grown to an optical density at 600 nm ($OD_{600}$) of 0.3 to 0.7 in CPG (~4 h). Cells were pelleted,

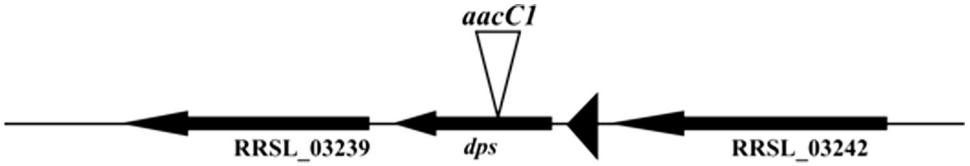


FIG. 1. Genomic context and mutagenesis of the gene coding for Dps in *R. solanacearum* strain UW551. The *dps* locus was disrupted by insertion of the *aacC1* gentamicin resistance gene cassette into a unique restriction site. In UW551, Dps is located 5′ of RRSL_03242, a LysR family regulator annotated in *R. solanacearum* GMI1000 as *oxyR*, a hydrogen peroxide-inducible gene activator. RRSL_03239 is annotated as *ubiA*, a prenyltransferase. The filled triangle represents a hypothetical protein.

washed, and adjusted to an $OD_{600}$ of 0.05 ($5 \times 10^7$ CFU/ml) in buffered BMM. To induce starvation in log-phase cells, overnight cultures were diluted and grown to an $OD_{600}$ of 0.3 to 0.7 in buffered BMM supplemented with 0.2% glucose. Cells were then collected by centrifugation, washed, and adjusted to an $OD_{600}$ of 0.01 ($1 \times 10^7$ CFU/ml) in buffered BMM supplemented with only 1% tomato root exudate (12) and incubated without aeration at room temperature for 2 days. Warm BMM top agar combined with a 100-μl bacterial suspension was poured over warm BMM plates to generate a bacterial lawn. Filter paper disks saturated with $H_2O_2$ at concentrations of 0, 250, and 500 mM were placed in the center of plates after the top agar solidified. After incubation for 48 h at 28°C, the diameter of each growth inhibition zone was measured twice along perpendicular axes. Each strain was tested in at least two independent assays with three individual disks per $H_2O_2$ treatment.

**Virulence assays.** The virulence of the *dps* mutant was measured by inoculating plants of the susceptible tomato cultivar 'Bonny Best' using a naturalistic soil soak method or by direct inoculation through a cut petiole as described previously (43). Unwounded 19-day-old tomato plants were inoculated by pouring bacterial suspension directly into pots to a final concentration of $\sim 1 \times 10^8$ CFU/g soil. For petiole inoculations, ~200 cells were applied directly to the cut petiole of the first true leaf of 21-day-old tomato plants. Bacterial wilt progress was rated daily according to the following disease index (DI): 0, healthy; 1, 1 to 25% leaf area wilted; 2, 26 to 50% leaf area wilted; 3, 51 to 75% leaf area wilted; and 4, >75% leaf area wilted. Each assay was replicated three times with 16 plants per treatment.

**Plant colonization studies.** To measure host colonization by *R. solanacearum* strains, we petiole inoculated 21-day-old tomato plants as described above. At 12, 24, 48, and 96 h after inoculation, we sampled approximately 1 cm of tomato stem bracketing the inoculation point. The stem segment was weighed, ground in 1 ml of sterile water, and dilution plated onto CPG, amended with antibiotic where necessary; colonies were counted after 48 h of incubation at 28°C. Each experiment included 10 plants per time point per treatment.

To determine relative competitive fitness of strains, 19-day-old tomato plants were soil soak inoculated with a 1:1 mixture of wild-type UW551rif and the UW551*dps* mutant strain to a final combined concentration of $5 \times 10^7$ CFU/g soil. Bacterial populations in plant stems were determined when tomato plants developed wilting symptoms or 22 days after inoculation, whichever came first. Bacterial population sizes in tomato stem tissue were determined as described above, except that aliquots were plated on both CPG Rif and CPG Kan plates to independently determine the CFU/g of each strain. Thirteen coinoculated plants were assessed in this assay.

**Determining stability of the UW551*dps* phenotype *in planta*.** Nineteen-day-old tomato plants were soil soak inoculated with $5.0 \times 10^7$ CFU/g soil of either UW551 or UW551*dps*. Bacterial populations in stems were determined from two wilted plants (DI = 1 to 3) for each treatment, and bacteria were isolated on CPG medium without selection. The resulting isolates were patched to CPG amended with gentamicin to determine if the antibiotic resistance gene cassette in the mutagenesis construct was still present. Three isolates each of strains UW551 and UW551*dps* recovered from wilted plants (named UW551′ and UW551*dps*′) and three cultures of UW551 and UW551*dps* grown from stocks were grown overnight in CPG, adjusted to $1.5 \times 10^9$ CFU/ml in BMM, and assayed for $H_2O_2$ sensitivity as described above. All six strains were also petiole inoculated into 10 susceptible tomato plants, and disease progress was monitored as described above. Each strain was then recultured on CPG plates and reassayed for $H_2O_2$ sensitivity.

**Mutation rate.** To measure the spontaneous mutation rate in late stationary-phase cells, cultures of UW551 and UW551*dps* were grown for 3 days in CPG, and rifampin plates were spread with 100-μl aliquots containing $5 \times 10^7$ to $7.5 \times 10^8$ cells, depending on the strain. All plates were incubated at 28°C for 4 days. Mutation frequency was calculated by dividing the number of rifampin-resistant colonies by the total cell number plated.

**Mutagenesis of *oxyR*.** A UW551 *oxyR* mutant was created by modified natural transformation (6). Briefly, overnight broth cultures of UW551 were diluted in fresh CPG and grown to an $OD_{600}$ of 0.5 to 0.9. Fifty microliters of bacterial suspension was incubated with 20 to 40 ng of genomic DNA from K60*oxyR* ($Gm^r$), a related *R. solanacearum* strain already carrying the desired mutation (Z. Flores-Cruz and C. Allen, unpublished data). A $Gm^r$ transformant was named UW551*oxyR*. The mutation was confirmed by PCR analysis and by phenotype (absence of catalase activity).

**Gene expression analysis.** Overnight broth cultures of *R. solanacearum* strains UW551 and UW551*oxyR* were adjusted to similar cell densities in fresh CPG and grown in triplicate to log phase or stationary phase. Bacterial suspensions were treated with 1.25 ml of 5% water-saturated phenol in ethanol per 10 ml of culture to preserve the RNA profile. Cells were collected by centrifugation and lysed with 1.0 mg/ml lysozyme (Fisher Scientific, Hanover Park, IL) and 80 μl 10% sodium dodecyl sulfate (Ambion, Foster City, CA) for 2 min at 65°C. RNA was extracted using a hot phenol-chloroform method (23). RNA concentration and purity was quantified using microspectrophotometry (NanoDrop 1000; Thermo Scientific, Wilmington, DE). cDNA was synthesized from 500 ng RNA with the SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (qRT-PCR) analysis was performed in duplicate in 25-μl volumes with 50 ng cDNA, 100 nM each primer, and 12.5 μl Power SYBR green PCR master mix (Applied Biosystems, Warrington, United Kingdom) using an ABI 7300 real-time PCR system (Applied Biosystems). Primers *dps* F (5′-GCC GATACCTACTCGCTCTATC-3′), *dps* R (5′-GTCTCGAACATCAGATGCA G-3′), *rplM* F (5′-CCGCAAAGCCCCATGAG-3′), and *rplM* R (5′-TGTCCGT CGCGTCAATCA-3′) were used to quantify *dps* expression by comparison with the stably expressed gene *rplM*, which encodes ribosomal protein L13, a component of the 50S ribosomal subunit. Results were averaged from three independent RNA extractions for each strain. The comparative threshold cycle ($C_T$) method was used to determine relative transcript abundance (28).

**Statistical analysis.** Data analyses were conducted using JMP 8.0 (SAS Institute, Raleigh, NC) or Minitab release 14.13 (State College, PA). Virulence assay results were analyzed by repeated measures analysis of variance (ANOVA), and other data were analyzed by ANOVA with Tukey's honestly significant difference test, the Mann-Whitney test, or paired *t* test.

## RESULTS

**Identification and mutagenesis of *dps* in *R. solanacearum*.** R3bv2 strain UW551 locus RRSL_03240 was identified in a promoter-trapping screen for genes induced by tomato root exudates (12). This screen generated a *dps*::*gus* reporter gene fusion strain (UW551*rex5*) that was upregulated about 4-fold in the presence of tomato root exudates (12). Bioinformatic analysis of RRSL_03240 and its chromosomal context (Fig. 1) suggested it could encode a homologue of the stress protective protein Dps (2). This UW551 locus has 99% amino acid identity to the corresponding protein in *R. solanacearum* strain GMI1000, and 28, 31, and 75% identity to the Dps proteins of *E. coli*, *A. tumefaciens*, and *P. putida*, respectively. UW551 Dps contains H51, H63, D77, and E81, the conserved amino acids of the essential ferroxidase center (9).

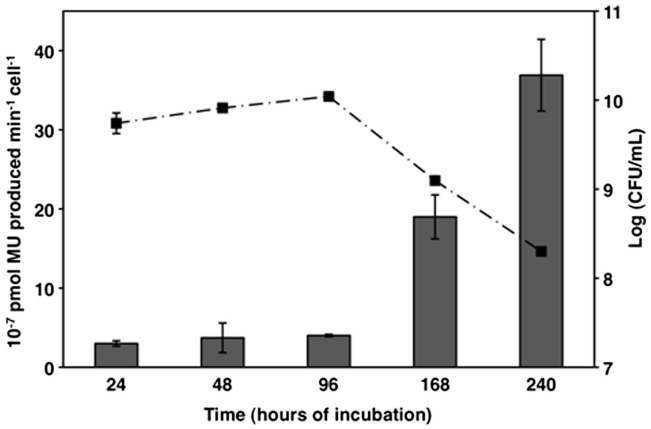


FIG. 2. Expression of *R. solanacearum dps* increased during prolonged stationary phase. An overnight culture of a *dps*::*gus* fusion strain was adjusted to $1 \times 10^7$ CFU/ml in buffered CPG broth, and expression of *dps* was measured over time as β-glucuronidase (Gus) activity (gray columns). After 7 and 10 days, *dps* expression increased as the culture underwent nutrient stress, as indicated by a declining population size (dashed line). Bars, standard errors of the means.

To test the hypothesis that *dps* is upregulated when *R. solanacearum* experiences nutrient and oxidative stress, we measured expression of a UW551*rex5 dps*::*gus* fusion in progressively starved and stressed bacteria growing at 28°C in buffered rich media over a period of 10 days. While there was little change in *dps* expression after 24, 48, and 96 h of incubation, after 168 and 240 h, expression increased 6- and 12-fold, respectively (Fig. 2). We observed no increases in *dps* expression following addition of 10, 25, or 50 mM $H_2O_2$. However, addition of 125, 250, and 500 mM $H_2O_2$ to cultures of UW551*rex5 dps*::*gus* resulted in high methyl umbelliferone fluorescence, indicating Gus activity, but this activity could not be quantified because we could not recover viable cells (data not shown).

To further investigate the role of *dps* in *R. solanacearum* UW551 fitness and virulence, we created a site-directed mutant strain, UW551*dps* (Fig. 1). Growth of UW551*dps* was indistinguishable from that of the wild type in rich media over a 10-day growth curve (data not shown). The mutant had a moderate growth defect in minimal medium, multiplying at 77% the rate of its wild-type parent, diverging from the wild type by mid-stationary phase (42 h). Addition of the wild-type *dps* gene in *trans* fully complemented this growth defect (data not shown).

**Loss of *dps* increased the mutation rate.** To determine whether Dps protects *R. solanacearum* DNA from damage when cells are under nutrient stress conditions, we plated 3-day-old nutrient-stressed cultures onto CPG supplemented with rifampin to compare the frequency of mutation to rifampin resistance in UW551 and UW551*dps*. The $6.0 \times 10^{-8}$ mutation frequency of UW551*dps* was significantly higher than that of wild-type UW551 at $8.1 \times 10^{-9}$ ($P < 0.001$; 2-sample *t* test).

**UW551*dps* was hypersensitive to ROS when under nutrient stress conditions.** The *dps* mutant strain was significantly more sensitive to 500 mM $H_2O_2$ stress than the wild-type strain when cells were in stationary phase ($P = 0.009$), and in starved log-phase cells ($P = 0.033$) (Table 2). We noted that starved log-phase cells of all strains were less tolerant of $H_2O_2$ stress than cells stressed by stationary phase, but the *dps* mutant had consistently lower ROS tolerance under both conditions, indicating that starvation was not necessary for expression of the *dps* phenotype. A wild-type copy of *dps* in *trans* restored UW551*dps* to wild-type $H_2O_2$ tolerance under all conditions tested. Similar results were observed in response to 250 mM $H_2O_2$ (data not shown).

**Dps contributed to tomato root adherence, stem colonization, and bacterial wilt disease development.** Adhesion to host roots is a critical early step in bacterial wilt pathogenesis, and the root surface is known to be oxidatively stressful. To determine whether Dps played a role in *R. solanacearum* attachment to host roots, we quantified the number of bacteria adhering to tomato seedling roots following incubation in a suspension of either wild-type UW551 or the *dps* mutant strain. After 1.5 h, adhering populations of UW551 were almost 9-fold larger than those of UW551*dps* ($P = 0.051$) (Fig. 3A). Attachment to host roots is one of the earliest steps in bacterial wilt pathogenesis, so we hypothesized that the *dps* mutant might also be required for full virulence. Susceptible tomato plants inoculated with UW551 in a naturalistic soil soak virulence assay showed a modest but significant delay in the development of bacterial wilt symptoms relative to those inoculated with the wild-type parent ($P = 0.039$) (Fig. 4A), although all plants succumbed to bacterial wilt disease by the end of the assay.

Unexpectedly, UW551*dps* was more reduced in virulence when ~200 bacteria were introduced directly into tomato vasculature through a cut petiole. Under these conditions, UW551*dps* could not kill all plants by 14 days postinoculation ($P < 0.0001$) (Fig. 4B). To measure the contribution of Dps to host colonization separately from root infection, we inoculated cut petioles with ~170 cells of either wild-type UW551 or UW551*dps* and quantified bacterial populations in stems over the next several days. At every time point, populations of UW551*dps* in these directly inoculated stems were significantly smaller than those in stems inoculated with the wild-type parent ($P \leq 0.03$) (Fig. 3B). For example, 12 h after inoculation, wild-type populations were almost 18-fold larger, with an average population size of $2.61 \times 10^3$ CFU/g stem tissue, while the *dps* mutant population averaged $1.48 \times 10^2$ CFU/g stem tissue.

TABLE 2. Hydrogen peroxide tolerance of *R. solanacearum* strains, as determined by disk inhibition assay

| Strain | Zone of inhibition (cm ± SE) at[a]: | |
|---|---|---|
| | Stationary phase[b] | Starved log phase[c] |
| UW551(pUFJ10) | 2.5 ± 0.10† | 5.6 ± 0.05† |
| UW551*dps*(pUFJ10) | 3.1 ± 0.04‡ | 6.1 ± 0.03‡ |
| UW551*dps*(pUF*dps*) | 2.4 ± 0.09† | 5.7 ± 0.04† |

[a] Filter disks containing 10 μl 500 mM hydrogen peroxide solution were placed on a minimal medium plate with a top agar overlay containing bacterial suspension. Zones of growth inhibition were measured following incubation for 48 h at 28°C. Values within each treatment followed by different symbols were significantly different ($P < 0.05$).

[b] Strains were grown for 2 days in rich medium. Cell densities were $>2 \times 10^9$ CFU/ml.

[c] Strains were grown for 2 days in minimal medium without glucose but supplemented with 1% tomato root exudate. Cell densities were $2 \times 10^7$ to $5 \times 10^7$ CFU/ml.

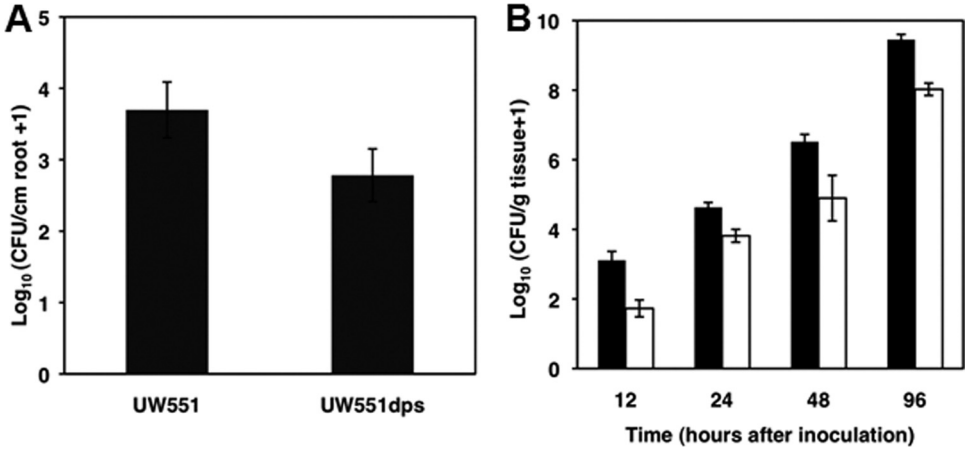


FIG. 3. A *Ralstonia solanacearum dps* mutant had reduced ability to adhere to tomato roots and colonize tomato stems. (A) Roots of tomato seedlings were incubated for 1.5 h in 6-well sterile tissue culture plates containing a suspension of $1.0 \times 10^7$ CFU/ml of either UW551 or UW551*dps*. Roots were rinsed, blotted, measured, ground, and dilution plated to enumerate adhering bacteria per cm of root. Average adhering populations of UW551*dps* were significantly lower than those of UW551 ($P = 0.05$). Bars, standard error of the mean. (B) Bacterial population sizes in tomato stems following cut petiole inoculation of 21-day-old plants with ~170 cells of either UW551 (black columns) or UW551*dps* (white columns). Plant stem samples were ground and dilution plated at intervals to enumerate bacteria. Ten plants were analyzed per time point per strain. Colonization levels differed significantly between strains at all time points ($P \leq 0.03$). Bars, standard errors of the means.

**Plant environment adapts *R. solanacearum* to $H_2O_2$ stress.** Wild-type *R. solanacearum* causes disease faster and with a lower level of inoculum when it is introduced directly into host stems via a cut petiole than when it must infect unwounded plants through the roots following soil soak inoculation (44). Our curious finding that the *dps* mutant was less virulent in a cut petiole assay than in a soil soak assay suggested that *R. solanacearum* undergoes a Dps-mediated adaptation to the stresses of the plant environment during host invasion and colonization. To test this hypothesis, we compared the ROS sensitivity of *R. solanacearum* cells grown in culture with cells isolated directly from infected tomatoes. Isolates of UW551 and UW551*dps* recovered from wilted plants were subjected to the $H_2O_2$ disk inhibition assay. As expected, UW551*dps* strains isolated from wilted plants (UW551*dps′*) were less tolerant of $H_2O_2$ than plant-isolated UW551 strains (UW551′). However, wild-type and *dps* mutant strains were unexpectedly both more tolerant of $H_2O_2$ after passage through plants than UW551 and UW551*dps* strains grown from culture ($P < 0.0001$) (Table 3). The two strains retained their relative differences in oxidative stress tolerance, but in both cases their absolute tolerance increased following exposure to the plant environment. When plant-passaged strains were reassayed for $H_2O_2$ tolerance following growth in rich medium, all strains had returned to their former lower tolerance, but the relative differences between the wild-type and *dps* mutant strains remained the same (Table 3).

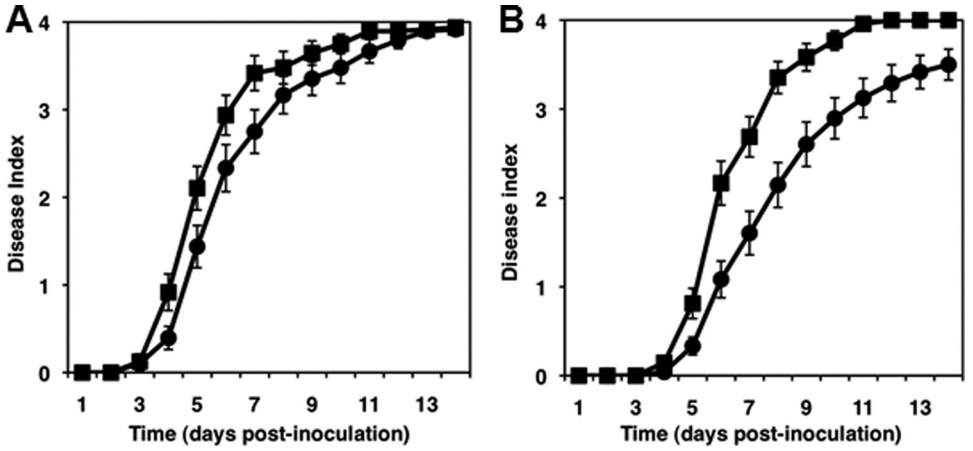


FIG. 4. Virulence of *Ralstonia solanacearum* wild-type strain UW551 and a *dps* mutant strain on tomato, measured by two different assays. (A) Naturalistic soil soak inoculation of unwounded 19-day-old 'Bonny Best' tomato plants with either UW551 (squares) or UW551*dps* (circles); disease progress of the *dps* mutant was slightly delayed relative to that of the wild type ($P < 0.032$; repeated measures ANOVA). (B) Cut petiole inoculation of 21-day-old plants with either UW551 (squares) or UW551*dps* (circles); the *dps* mutant was less virulent than the wild type ($P < 0.0001$; repeated measures ANOVA). Plants were rated daily on a disease index scale of 0 to 4; points represent the mean disease index of three independent assays with 16 plants per treatment per assay. Bars, standard errors of the means.

TABLE 3. Hydrogen peroxide tolerance of *R. solanacearum* strains UW551 and UW551*dps* recovered from wilted tomato plants and grown from culture, as determined by disk inhibition assay

| Strain[b] | Zone of inhibition (cm ± SE) at[a]: | |
|---|---|---|
| | 6 days (initial)[c] | 9 days (recultured)[d] |
| UW551 wild type (culture) | 2.6 ± 0.07† | 3.0 ± 0.09† |
| UW551*dps* (culture) | 3.0 ± 0.05‡ | 3.5 ± 0.11‡ |
| UW551′ wild type (wilted plant) | 2.1 ± 0.02§ | 2.7 ± 0.08§ |
| UW551*dps*′ (wilted plant) | 2.6 ± 0.02† | 3.0 ± 0.11† |

[a] Filter disks containing 10 μl 3% hydrogen peroxide solution were placed on a plate with a top agar overlay containing bacterial suspension. Zones of growth inhibition were measured following incubation for 48 h at 28°C. Values within each treatment followed by different symbols were significantly different ($P < 0.05$).

[b] Strains were either cultured directly onto rich medium (culture) or recovered from wilted tomato plants (represented by ′).

[c] Assay performed on bacterial cells 6 days either after being transferred from culture plates or after isolates were recovered from wilted plants.

[d] Assay performed 3 days after all strains were recultured in rich medium.

***dps* is positively regulated by OxyR.** Based on the proximity of *dps* to the *oxyR* locus in the UW551 genome, and a predicted *oxyR* transcriptional activation motif upstream of *dps*, we tested the hypothesis that *dps* transcription is modulated by the global oxidative stress response regulator OxyR. We used quantitative real-time PCR analysis to measure *dps* expression in a UW551*oxyR* genetic background relative to the wild type during both log and stationary growth phases. Our results suggest that in the exponential growth phase, OxyR is necessary for wild-type levels of *dps* expression, as indicated by a 2.6-fold reduction in *dps* transcript ($P \leq 0.0001$). In contrast, when cells were in stationary phase, there was no difference in *dps* expression between the wild-type and *oxyR* strains ($P = 0.428$).

## DISCUSSION

Early in its disease cycle, *R. solanacearum* undergoes a metabolic transition from soil survivor to plant colonist. The pathogen must adapt to the specific stresses that confront it as it traverses the host rhizosphere and enters plant tissue. These stresses likely include ROS and starvation or competition for nutrients. Once the pathogen colonizes its host it is likely confronted with higher levels of ROS as well as other host defensive antimicrobial compounds, such as phytoalexins.

Tomato root exudates, which appear to signal to the pathogen that it is in a host rhizosphere (49), cause *R. solanacearum* to upregulate expression of the Dps stress survival protein. A *dps*::*gus* reporter fusion was highly expressed under all conditions tested, but it was even further induced when *R. solanacearum* cells were starved. Consistent with these results, a global microarray analysis of UW551 gene expression found that Dps is among the most highly expressed proteins of *R. solanacearum*, both when bacteria were at late log phase in rich medium and at comparable cell densities during tomato pathogenesis (L. Babujee, J. Jacobs, F. Meng, and C. Allen, unpublished results). However, *dps* expression did not increase when *dps*::*gus* fusion strains in stationary phase were exposed to the relatively low concentration of ≤50 mM $H_2O_2$ stress (data not shown), and the *dps* mutant in stationary phase of growth survived an hour of exposure to 25 mM $H_2O_2$ as well as its wild-type parent (data not shown). This result is consistent with the finding that an *E. coli dps* mutant survived <10 mM $H_2O_2$ as well as its wild-type parent (29). Thus, low concentrations of $H_2O_2$ do not appear to regulate expression of *dps* in *R. solanacearum*, and the Dps protein does not contribute measurably to tolerance of low concentrations of hydrogen peroxide. This could be attributed to the length of time or intensity of ROS exposure. Consistent with this idea, stationary phase and starved *dps* cells did have decreased ROS tolerance in disk inhibition plate assays, which expose bacteria to high hydrogen peroxide concentrations.

Dps is believed to protect prokaryotes from stress by means of a conserved ferroxidase center. At this site, iron is incorporated and reduced, decreasing the hydroxyl radicals produced in the Fenton reaction when iron interacts with hydrogen peroxide (9, 34). Our finding that Dps contributes to oxidative stress tolerance during starvation suggests that this protein helps *R. solanacearum* survive the challenging environmental conditions in the rhizosphere and, possibly, in the later stages of tomato stem colonization when nutrients are becoming depleted. The high basal expression level of this gene, together with the slight growth defect of UW551*dps* in minimal medium, indicate that this protein plays a core role in *R. solanacearum* fitness as well as in conferring tolerance to specific additional stresses. The *dps* mutant also had reduced viability on plates following culture to low cell densities or when it was suspended in water or minimal medium (data not shown). Adding catalase to plates allowed recovery of wild-type CFU numbers, demonstrating that poor ROS tolerance was responsible for this phenotype. However, we noticed that over several hours in water the *dps* mutant populations appeared to recover viability (data not shown), consistent with the idea that *R. solanacearum* has multiple mechanisms for stress adaptation.

We found that Dps contributes to ROS tolerance of nutrient-stressed *R. solanacearum* cells, both in stationary-phase cultures and when dilute populations are starved. In stationary-phase *E. coli*, Dps binds to DNA to form very stable complexes and reduces the frequency of base pair mutations (2, 29). While Dps appears to enable $H_2O_2$ stress tolerance broadly among prokaryotes, the mechanisms of Dps function may differ in soilborne plant-associated bacteria. For example, in *A. tumefaciens*, Dps does not bind to DNA, although the residues responsible for DNA binding in *E. coli* Dps are conserved (9). Eleven of the 12 DNA-binding residues identified in *A. tumefaciens* and *E. coli* are found in *R. solanacearum* UW551 (9). Furthermore, in *P. putida*, Dps does not affect mutation accumulation in starving cells, while *E. coli dps* mutants have increased specific base substitution rates; moreover, expressing *dps* from an inducible plasmid reduced the frequency of spontaneous base substitutions in the wild type (29, 37). Our finding that UW551*dps* had an increased frequency of spontaneous mutation suggests that Dps confers some protection from mutation, but the mechanism is unknown. Experiments to determine whether *R. solanacearum* Dps binds DNA directly could shed some light on the mechanism involved.

The virulence of an *R. solanacearum dps* mutant was lower on tomato in both a biologically relevant soil soak inoculation assay and in a more artificial cut petiole inoculation assay.

Unexpectedly, the mutant was more significantly reduced in virulence in the cut petiole assay than in the soil soak assay. The small delay in virulence in the soil soak assay could be explained in part by the reduced ability of the *dps* mutant to adhere to tomato roots and a general fitness defect, suggested by its slightly slower growth in minimal medium. The decreased ability to adhere to tomato roots was probably not due to nutrient or osmotic stress, because the cells used in the adhesion assay had not been starved and the *dps* mutant survived as well as the wild type under the osmotic conditions tested (data not shown). The reduced ROS tolerance of the *dps* mutant may explain its reduced adhesion, since the root surface is known to be an oxidative environment (32). However, it was puzzling that the *dps* mutant was more substantially decreased in virulence in the cut petiole assay, which usually places fewer demands on the pathogen. We speculate that during the natural infection process, Dps helps the bacterium adapt to conditions inside the host but that, given time, other mechanisms of adaptation can compensate to some degree for the loss of Dps function. When cells were introduced directly into the host xylem vessels without experiencing the conditioning effects of the rhizosphere and root colonization, the *dps* mutant suffered a more acute disadvantage.

Experiments intended to test this conjecture revealed that both wild-type and *dps* mutant *R. solanacearum* cells had increased tolerance to $H_2O_2$ after passage through tomato plants. This plant-induced increased ROS tolerance persisted for several days in culture, but eventually diminished. Several of the numerous oxidative stress response genes of *R. solanacearum* are upregulated during growth in host plants (8, 14). These may provide extended protection to the pathogen after it is shed from a diseased plant, increasing its ability to survive in the soil or in association with plant debris. We cannot at present propose a mechanism by which the bacteria retain their ROS-tolerant state over multiple divisions, but as both wild-type and *dps* mutant strains exhibited this behavior, it must be independent of Dps. Identification of these additional Dps-independent mechanisms of plant adaptation is needed to understand this key habitat transition process. Returning to the problem of why the *dps* mutant had a greater virulence defect when introduced directly into host xylem than when inoculated via soil soaking, we can conclude only that the proposed Dps-mediated adaptation to growth inside plants must involve protection from a stress other than ROS.

Our data suggest that in the rhizosphere, an *R. solanacearum* population faces nutrient and/or oxidative stress, which affects its ability to attach to and colonize tomato roots early in the interaction. Plants produce ROS in the rhizosphere in response to many factors, including hormone signaling, root growth, gravitropism, and nodulation (5, 25, 32, 35). Furthermore, tomato xylem has been shown to be a highly oxidative environment, and *R. solanacearum* uses several independent pathways to protect itself from plant-derived reactive oxygen species (8, 14). These redundant oxidative stress response proteins may explain the ability of the *dps* mutant to overcome its deficiency and ultimately wilt the tomato host after soil soak inoculation.

In *E. coli*, *dps* is regulated differentially by growth phase. OxyR and the *rpoS*-encoded $\sigma^S$ activate *dps* expression in log- and stationary-phase cells, respectively (3). Quantitative PCR analysis in UW551 and UW551*oxyR* demonstrated that *R. solanacearum dps* expression was reduced in an *oxyR* background when cells were in exponential growth phase, but not when they were in stationary phase. This suggests that *dps* may be regulated similarly in *R. solanacearum* and in *E. coli*. Experiments to determine the role of RpoS in *dps* regulation could more fully define the relationship of cell density to *dps* expression. Some evidence suggests that in *R. solanacearum*, OxyR itself is cell density responsive (Flores-Cruz and Allen, unpublished results).

This study provides new insights into a phytopathogenic bacterium's interaction with its host and offers indirect information about the plant environment. Several questions remain unanswered, including whether Dps binds DNA and whether it protects DNA when *R. solanacearum* is in the xylem vessels of its plant host. It would be useful to determine whether, in addition to increasing ROS tolerance, Dps also contributes to tolerance of other environmental stressors such as osmotic, temperature, or pH stress. Furthermore, is the conditioning effect we see when R3bv2 passes through the host plant acquired in the rhizosphere, before host entry, or within the plant during active disease? This study suggests that *R. solanacearum* uses multiple tactics, especially partially redundant adaptation strategies, to persevere in a challenging environment.

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